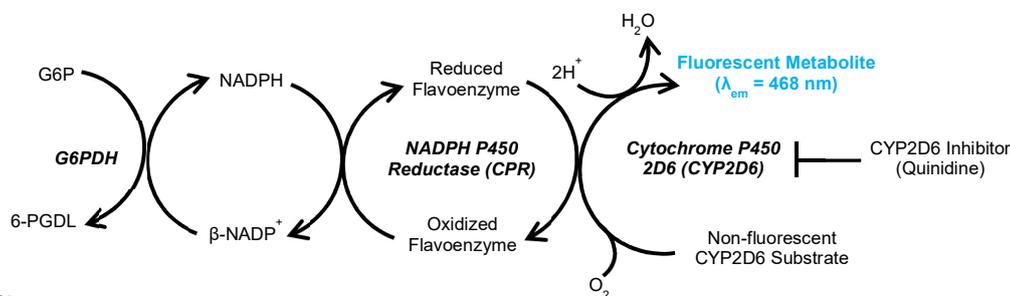


Cytochrome P450 2D6 (CYP2D6) Activity Assay Kit (Fluorometric)

(Catalog # BN00927; 200 Reactions; Store at -20°C)

I. Introduction:

Cytochrome P450 2D6 (CYP2D6, EC 1.14.14.1) is a member of the cytochrome P450 monooxidase (CYP) family of microsomal xenobiotic metabolism enzymes. CYPs are membrane-bound heme proteins responsible for Phase I biotransformation reactions, in which lipophilic drugs and other xenobiotic compounds are converted to more hydrophilic products to facilitate excretion from the body. CYP2D6 catalyzes oxidation of lipophilic bases with an aromatic ring and a nitrogen atom and is highly expressed in liver and brain tissue. The enzyme is responsible for metabolism of nearly 25% of all small molecule drugs commonly used by humans, particularly psychiatric drugs such as antidepressants, antipsychotics and stimulants. The CYP2D6 gene is highly polymorphic in the human population, with CYP2D6 activity ranging from complete metabolic deficiency to ultra-rapid metabolism. Due to this wide phenotypic variability, CYP2D6 is frequently implicated in drug toxicity and clinical drug/drug interactions. In addition, for drugs whose pharmacological activity requires metabolism from a pro-drug form, CYP2D6 inhibition or allelic deficiency can lead to decreased drug efficacy. Assay Genie's CYP2D6 Activity Assay Kit enables rapid measurement of native or recombinant CYP2D6 activity in biological samples such as liver microsomes. The assay utilizes a non-fluorescent CYP2D6-selective substrate that is converted into a highly fluorescent metabolite detected in the visible range (Ex/Em = 390/468 nm), ensuring a high signal-to-background ratio with little interference by autofluorescence. CYP2D6 specific activity is calculated by running parallel reactions in the presence and absence of the potent CYP2D6-selective inhibitor quinidine and subtracting any residual activity detected with the inhibitor present. The kit contains a complete set of reagents sufficient for performing 100 sets of paired reactions (in the presence and absence of inhibitor).



II. Applications:

- Rapid assessment of native/recombinant CYP2D6 activity in fractions prepared from tissues and cells.
- Screening of drugs and novel ligands for interaction with native/recombinant CYP2D6.

III. Sample Type:

- Human liver microsomes and liver S9 fractions
- Lysates of tissues and cultured cells, primary hepatocytes
- Heterologously expressed recombinant CYP2D6 preparations

IV. Kit Contents:

Components	BN00927	Cap Code	Part Number
CYP2D6 Assay Buffer	100 ml	NM	BN00927-1
AHMC Standard	1 vial	Yellow	BN00927-2
CYP2D6 Inhibitor (Quinidine)	1 vial	Amber	BN00927-3
NADPH Generating System (100X)	1 vial	Green	BN00927-4
β-NADP ⁺ Stock (100X)	1 vial	Blue	BN00927-5
CYP2D6 Substrate	1 vial	Red	BN00927-6
Recombinant Human CYP2D6	1 vial	Violet	BN00927-7

V. User Supplied Reagents and Equipment:

- Multiwell fluorescence microplate reader
- Precision multi-channel pipette and reagent reservoir
- Anhydrous (reagent grade) acetonitrile and DMSO
- Opaque white 96-well plates with flat bottom

VI. Storage Conditions and Reagent Preparation:

Store kit at -20°C and protect from light. Briefly centrifuge all small vials prior to opening. Allow the CYP2D6 Assay Buffer to warm to room temperature prior to use. Read entire protocol before performing the assay procedure.

- **AHMC Standard:** Reconstitute in 110 μl of DMSO and vortex until fully dissolved to yield a 2 mM stock solution. The AHMC stock solution should be stored at -20°C and is stable for at least 3 freeze/thaw cycles.
- **CYP2D6 Inhibitor (Quinidine):** Reconstitute in 220 μl of acetonitrile and vortex until fully dissolved to yield a 2 mM stock solution. The stock solution is stable for 2 months at -20°C. To obtain a 15 μM working solution of quinidine (5X final concentration), add 15 μl of the 2 mM stock solution to 1985 μl of CYP2D6 Assay Buffer. Store the 15 μM quinidine solution at -20°C and use within one week.
- **NADPH Generating System (100X):** Reconstitute with 440 μl CYP2D6 Assay Buffer, aliquot and store at -20°C. Avoid repeated freeze/thaw cycles and keep on ice while in use.

- **β -NADP⁺ Stock (100X):** Dissolve in 440 μ l CYP2D6 Assay Buffer and vortex thoroughly to yield a 100X stock. Store at -20°C, stable for at least 3 freeze/thaw cycles.
- **CYP2D6 Substrate:** Reconstitute with 220 μ l anhydrous reagent-grade acetonitrile and vortex until fully dissolved. Store at -20°C. Allow the vial to warm to room temperature before opening and promptly retighten cap after use to avoid absorption of airborne moisture.
- **Recombinant Human CYP2D6:** Do not reconstitute until ready to use. Reconstitute with 460 μ l CYP2D6 Assay Buffer and add 40 μ l of NADPH Generating System (100X). Mix thoroughly to ensure a homogenous solution, aliquot and store at -80°C. Avoid repeated freeze/thaw cycles and use aliquots within one month (the Recombinant Human CYP2D6 will lose approximately 10% activity per week when stored at -80°C). Thaw aliquots rapidly at 37°C and place on ice until use (thawed aliquots should be used within 4 hours).

VII. Cytochrome P450 2D6 (CYP2D6) Activity Assay Kit Protocol:

1. Standard Curve Preparation:

- Dilute the AHMC Standard (2 mM stock) by adding 5 μ l of the 2 mM solution to 995 μ l CYP2D6 Assay Buffer to generate a 10 pmole/ μ l (10 μ M) solution of AHMC. Add 0, 2, 4, 6, 8, 12, 16 and 20 μ l of the 10 pmole/ μ l AHMC standard into a series of wells in an opaque 96-well plate. Adjust the volume of each well to 100 μ l with CYP2D6 Assay Buffer, yielding a standard curve with 0, 20, 40, 60, 80, 120, 160 and 200 pmole/well of AHMC.
- Measure fluorescence at Ex/Em = 390/468 nm. Subtract the blank reading (0 nmole/well) from all of the standard readings, plot the background-subtracted values and calculate the slope of the standard curve.

2. Sample and Test Compound Preparation:

- Standardized microsomal preparations may be purchased commercially (e.g. donor-pooled human liver microsomes) or prepared from liver tissue or cultured cells using the Microsome Isolation Kit. Alternatively, a crude enriched lysate can be prepared: start with ~50 mg tissue or ~5 x 10⁶ pelleted, pre-washed cells and homogenize in 500 μ l ice-cold CYP2D6 Assay Buffer with a Dounce homogenizer on ice. Incubate the homogenate on ice for 5 min. and then centrifuge at 15,000 x g for 15 min. in a refrigerated centrifuge at 4°C. Collect the resultant clarified supernatant for the assay in a new pre-chilled microfuge tube and store on ice until use (cell and tissue lysates can also be stored at -80°C in aliquots for future experiments).
- If desired, CYP2D6 activity in presence of test ligands may be measured. Test ligands should be dissolved into proper solvent to produce stock solutions (see note regarding solvent effects below). For each ligand, prepare a 5X solution by diluting in CYP2D6 Assay Buffer.

Notes:

- To quantify CYP2D6 specific activity in terms of sample protein content, use the Bradford reagent or an equivalent protein assay.
- When measuring CYP2D6 activity in presence of ligands (inhibitors or substrates), run parallel solvent control well(s) to account for additional solvent in the reaction mix. Many commonly-used organic solvents can severely impact CYP2D6 activity. Importantly, DMSO causes significant inhibition of CYP2D6 at final concentrations $\geq 0.2\%$ (v/v). Our assay is designed to use acetonitrile at a final concentration of $\leq 1\%$, which has been shown to have little impact on CYP2D6 activity.

3. Reaction Preparation:

- Prepare enough reagents for the number of reactions to be performed. For each reaction, prepare a 2X concentrated P450 reaction mix by combining 2-48 μ l of sample and 2 μ l of the NADPH Generating System (100X) in a 96-well plate and adjusting the final volume to 50 μ l/reaction with CYP2D6 Assay Buffer. The amount of sample per reaction and the dilution factor required will vary based upon the nature of the sample. In human liver tissue, CYP2D6 typically accounts for only a small fraction of the total P450 content (approximately 2-4%). Hence, for human liver microsomes, we recommend starting with a final protein concentration of 0.5 mg/ml (50 μ g of microsomes per well). For liver S9 fractions or other cellular lysates, the amount of protein required will be significantly higher. In this case, we recommend starting at 2-4 mg/ml protein (100-200 μ g/well).
Note: Due to the large individual variation in CYP2D6 expression level and function, sample protein levels may need to be adjusted.
- In addition to the test samples, prepare background control (no enzyme) and inhibitor control (3 μ M quinidine) wells. If desired, you may also prepare CYP2D6 enzyme positive control wells using the Recombinant Human CYP2D6. Adjust the volume of test sample, inhibitor control and positive control wells to 70 μ l/well with CYP2D6 Assay Buffer. For measurement of CYP2D6 activity in the presence of test ligands, replace CYP2D6 Assay Buffer with 5X concentrated test ligand solution:

	Test Sample	+ Inhibitor Control	Background	Positive Control
P450 Reaction Mix (2X)	50 μ l	50 μ l	—	—
Recombinant Human CYP2D6	—	—	—	25 μ l
Quinidine 15 μ M Solution (5X)	—	20 μ l	—	—
CYP2D6 Assay Buffer (or Test Ligand 5X)	20 μ l	—	70 μ l	45 μ l

- Incubate the plate for 10-15 min at 37°C to allow the inhibitor quinidine or any test ligands to interact with CYP2D6 in the absence of P450 catalytic turnover. During the incubation, prepare a CYP2D6 Substrate/NADP⁺ mixture (3X) by adding 30 μ l of the reconstituted 2 mM CYP2D6 Substrate stock solution and 100 μ l of the reconstituted 5 mM β -NADP⁺ Stock (100X) to 2870 μ l of CYP2D6 Assay Buffer for a total volume of 3 ml. This preparation is sufficient for 100 reactions, but can be scaled depending upon the number of reactions to be performed.
- Start the reaction by adding 30 μ l of the CYP2D6 Substrate/NADP⁺ (3X) mixture to each well using a multichannel pipette, yielding a final reaction volume of 100 μ l/well.

Note: The Recombinant Human CYP2D6 preparation may settle and should be thoroughly mixed before dispensing.

- Measurement:** Immediately (within 1 min) measure the fluorescence at Ex/Em = 390/468 nm in kinetic mode for 60 min at 37°C. While the assay can be performed in either endpoint or kinetic mode, we strongly recommend reading in kinetic mode in order to ensure that the measurements recorded are within the linear range of the reaction. Ideal measurement time for the linear range may vary depending upon experimental conditions.

Note: Since the reaction starts immediately after the addition of the CYP2D6 Substrate/NADP⁺ mix, it is essential to preconfigure the fluorescence microplate reader settings and use a multichannel pipette with a reagent reservoir to minimize lag time among wells.

- 5. Calculation:** For each reaction well (including background and positive inhibition controls), choose two time points (T_1 and T_2) in the linear phase of the reaction progress curves, obtain the corresponding fluorescence values at those points (RFU_1 and RFU_2) and determine the change in fluorescence over the time interval: $\Delta F = RFU_2 - RFU_1$. Subtract the ΔF value of the background control (BC) from those of the test samples (S) and 3 μM quinidine positive inhibition control (Q) to determine the background-corrected change in fluorescence intensity for each well. Calculate the specific fluorescence generated by CYP2D6 activity (denoted by C) by subtracting the background-corrected positive inhibition control from each sample:

$$C_S = (\Delta F_S - \Delta F_{BC}) - (\Delta F_Q - \Delta F_{BC}) = (\Delta F_S - \Delta F_Q)$$

Note: In our experience, the CYP2D6 Substrate does not undergo appreciable non-enzymatic conversion to the fluorescent product during the course of a typical assay (60 min). Depending upon instrumental noise, the rate calculation for the no enzyme/background control (BC) well may yield a negative value, in which case, the BC value may be ignored.

CYP2D6 metabolic activity is obtained by applying the C_S values to the AHMC standard curve to get B pmole of substrate metabolized to AHMC by CYP2D6 during the reaction time.

$$\text{Cytochrome P450 2D6 Specific Activity} = \frac{B}{\Delta T \times P} = \text{pmole/min/mg} = \mu\text{U/mg}$$

Where: **B** is the amount of AHMC produced, calculated from the standard curve (in pmole)

ΔT is the linear phase reaction time $T_2 - T_1$ (in minutes)

P is the amount of protein in the sample (in mg)

CYP2D6 Unit Definition: One unit of CYP2D6 activity is the amount of enzyme that generates 1 μmole of AHMC per min by hydrolysis of 1 μmole fluorogenic substrate at 37°C and pH 8.0.

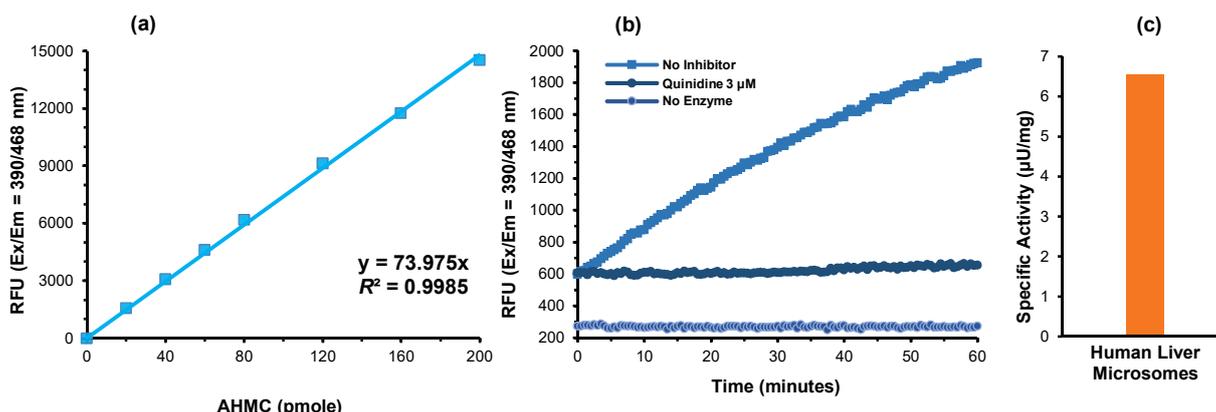


Figure: (a) AHMC standard curve. One mole of AHMC corresponds to the metabolism of one mole of CYP2D6 substrate. (b) Reaction kinetics of fluorogenic substrate metabolism in human liver microsomes (0.5 mg/ml) at 37°C in the presence and absence of the selective CYP2D6 inhibitor quinidine (the no inhibitor reaction contained a final concentration of 0.5% acetonitrile). (c) Specific activity of CYP2D6 in pooled human liver microsome sample (0.5 mg/ml). Assays were performed according to the kit protocol.

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